IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Philipp Hadwiger et al.
Serial No:	10/543,048
Confirmation No:	3878
Filed:	January 26, 2006
For:	LIPOPHILIC DERIVATIVES OF DOUBLE-STRANDED RIBONUCLEIC ACID
Examiner:	Chong, Kimberly
Art Unit:	1635

APPEAL BRIEF PURSUANT TO 37 C.F.R. § 41.37

Commissioner for Patents
Mail Stop Appeal Brief - Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

As set forth in the Notice of Appeal filed March 23, 2010, Appellants hereby appeal the examiner's final rejection of claims 86, 94-98, 100-102, 110-111, and 114-119 of the above-identified application.

Appellants respectfully request that the Board of Patent Appeals and Interferences reverse the rejection of these claims.

I. REAL PARTY IN INTEREST

The real party in interest is Alnylam Pharmaceuticals, the assignee of U.S. Patent Application Serial No. 10/543,048. The assignment from the inventors to Alnylam Europe AG was recorded in the U.S. Patent and Trademark Office on Reel/Frame 017216/0009 on January 26, 2006, and the assignment from Alnylam Europe AG to Alnylam Pharmaceuticals was recorded in the U.S. Patent and Trademark Office on Reel/Frame 023120/0064 on August 19, 2009.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences pertaining to the above-identified application.

III. STATUS OF CLAIMS

Claims 86, 94-98, 100-102, 110-111, and 114-119 are currently pending. Claims 1-85, 87-93, 99, 103-109, and 112-113 have been cancelled.

Claims 86, 100-102, 110-119 have been rejected in the Office Action mailed November 12, 2009 under 35 U.S.C. § 103(a) as being unpatentable over U.S. Patent Application Publication No. 2003/0139363 to Kay et al. ("Kay") in view of U.S. Patent Application Publication No. 2003/0143732 to Fosnaugh et al. ("Fosnaugh"), U.S. Patent No. 7,097,856 to Frecht et al. ("Frecht"), and the 2000 Journal of Controlled Release article authored by Florence et al. ("Florence"). Claims 86, 94-98, and 110-119 have also been rejected as being unpatentable over Kay in view of Fosnaugh, U.S. Patent Application Publication No. 2003/0064492 to Manoharan et al. ("Manoharan I"), and U.S. Patent No. 6,803,198 to Cook et al. ("Cook"), as evidenced by the Manoharan reference cited in Appellants' IDS submitted on February 13, 2006, presumably the 2002 Antisense and Nucleic Acid Drug Development article by Manoharan et al. ("Manoharan II").

The decision of the examiner rejecting claims 86, 94-98, 100-102, 110-111, and 114-119 is hereby appealed. Claims 86, 94-98, 100-102, 110-111, and 114-119 are set forth in the attached Claims Appendix.

IV. STATUS OF AMENDMENTS

There are no amendments pending. All previous amendments and new claims were entered by the examiner.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claim 86 is directed to a double-stranded ribonucleic acid (dsRNA) comprising a complementary RNA strand, a sense RNA strand and only one lipophilic group having a $\log K_{ow}$ exceeding 1. The complementary RNA strand has a nucleotide sequence which is complementary to a target RNA. The target RNA is a mRNA transcript of a target gene or of a (+) strand RNA virus. The lipophilic group is covalently attached to a 5'-end of the complementary RNA strand and a linkage between the lipophilic group and the 5'-end of the complementary RNA strand comprises a phosphodiester group. Support for claim 1 may be found in original claims 1-4 and 24, and pages 4-5 of the specification.

Claim 94 is directed to the dsRNA of claim 86, wherein the lipophilic group is a steroid or a branched aliphatic hydrocarbon, or a combination thereof. Support for this claim may be found in the specification on page 7, lines 12-15 and page 14, line 27 to page 15, line 22.

Claim 95 is directed to the dsRNA of claim 94, wherein the lipophilic group is a sterol. Support for this claim may be found in the specification on page 15, lines 15-22.

Claim 96 is directed to the dsRNA of claim 95, wherein the sterol is cholesterol or a cholesterol derivative. Support for this claim may be found in the specification on page 15, lines 15-22.

Claim 97 is directed to the dsRNA of claim 96, wherein the lipophilic group is cholesteryl (6-hydroxyhexyl) carbamate or 12-hydroxydodecanoic acid bisdecylamide. Support for this claim may be found in the specification on page 14, line 27 to page 16, line 22.

Claim 98 is directed to the dsRNA of claim 86, wherein the lipophilic group is selected from the group consisting of an aromatic, aliphatic or alicyclic moiety, or a combination thereof. Support for this claim may be found in the specification on page 14, line 27 to page 17, line 1.

Claim 100 is directed to the dsRNA of claim 86, wherein the lipophilic group has a $\log K_{ow}$ exceeding 1.5. Support for this claim may be found in the specification on page 13, lines 3-21.

Claim 101 is directed to the dsRNA of claim 86, wherein the lipophilic group has a $\log K_{ow}$ exceeding 2. Support for this claim may be found in the specification on page 13, lines 3-21.

Claim 102 is directed to the dsRNA of claim 86, wherein the lipophilic group has a $\log K_{ow}$ exceeding 3. Support for this claim may be found in the specification on page 13, lines 3-21.

Claim 110 is directed to the dsRNA of claim 86, wherein the complementary RNA strand comprises a 3'-end and a 5'-end, and wherein the 3'-end has a nucleotide overhang of 1 to 4 nucleotides. Support for this claim may be found in the specification on page 16, lines 16-29.

Claim 111 is directed to the dsRNA of claim 86, wherein the complementary RNA strand comprises a 3'-end and a 5'-end, and wherein the 3'-end has a nucleotide overhang of 1 or 2 nucleotides. Support for this claim may be found in the specification on page 16, lines 16-29.

Claim 114 is directed to the dsRNA of claim 86, wherein the dsRNA is between 16 and 30 nucleotides in length. Support for this claim may be found in the specification on page 16, lines 9-15.

Claim 115 is directed to the dsRNA of claim 86, wherein the dsRNA is between 16 and 25 nucleotides in length. Support for this claim may be found in the specification on page 16, lines 9-15.

Claim 116 is directed to the dsRNA of claim 86, wherein the dsRNA is between 20 and 25 nucleotides in length. Support for this claim may be found in the specification on page 16, lines 9-15.

Claim 117 is directed to the dsRNA of claim 86, wherein the target RNA is expressed in a cell selected from the group consisting of a hepatocyte, a pancreatic cell, a uterine cell, a cell of a cervix, and a cell of a urinary bladder. Support for this claim may be found in the specification on page 9, lines 19-25, and original claim 66.

Claim 118 is directed to the dsRNA of claim 86, wherein the (+) strand RNA virus is a Hepatitis C Virus (HCV). Support for this claim may be found in the specification on page 9, lines 19-25, and original claim 67.

Claim 119 is directed to the dsRNA of claim 86, wherein the target RNA is at least a portion of a 3'-untranslated region (3'-UTR) of a Hepatitis C Virus (HCV). Support for this claim may be found in the specification on page 9, lines 19-25 and original claim 68.

VI. GROUNDS OF REJECTION TO BE REVIEWED UPON APPEAL

Appellants respectfully request that the Board reverse the rejection of claims 86, 100-102, 110-111, and 114-119 under 35 U.S.C. § 103(a) as being unpatentable over Kay in view of Fosnaugh, Frecht, and Florence, and reverse the rejection of claims 86, 94-98, 110-111, and 114-119 under 35 U.S.C. § 103(a) as being unpatentable over Kay in view of Fosnaugh, Manoharan I, and Cook, as evidenced by Manoharan II.

VII. ARGUMENT

a. Rejection of claims 86, 100-102 and 110-119 under 35 U.S.C. § 103 over the combination of Kay, Fosnaugh, Frechet, and Florence

The examiner has rejected claims 86, 100-102, and 110-119 under 35 U.S.C. § 103(a) as being unpatentable over Kay in view of Fosnaugh, Frecht, and Florence.

Claim 86 recites a double-stranded ribonucleic acid (dsRNA) comprising a complementary RNA strand, a sense RNA strand and only one lipophilic group having a $\log K_{ow}$ exceeding 1. The complementary RNA strand has a nucleotide sequence which is complementary to a target RNA, and wherein the target RNA is an mRNA transcript of a target gene or of a (+) strand RNA virus. The lipophilic group is covalently attached to a 5'-end of the complementary RNA strand and a linkage between the lipophilic group and the 5'-end of the complementary RNA strand comprises a phosphodiester group.

Kay is cited for the teaching of dsRNA that efficiently inhibit viral gene expression, and targeting hepatocyte cells using a dsRNA molecule capable of inhibiting the expression of a Hepatitic C Virus. See Office Action mailed March 19, 2009, page 4. Kay does not teach a

lipophilic group linked at the 5' end with a phosphodiester group. To cure these deficiencies, the examiner relies heavily on Fosnaugh. Specifically, the examiner cites Fosnaugh as teaching a dsRNA that comprises a conjugate covalently attached to the dsRNA, with broad language suggesting that the conjugate may be attached to either end of either strand. The examiner also relies on Fosnaugh for teaching that the conjugate can be linked with biodegradable linkers and phosphodiester linkages. See Office Action mailed March 19, 2009, page 4. Frechet has been cited by the examiner as disclosing dendrimers that can be conjugated to nucleic acids, including dsRNA. See Office Action mailed March 19, 2009, pages 4-5. Florence allegedly discloses lipophilic dendrimers having an octanol/water coefficient of 17.5. See Office Action mailed March 19, 2009, page 5.

The examiner, however, has not produced a single reference showing a dsRNA linked to a lipophilic conjugate (having a $\log K_{ow}$ exceeding 1) via a phosphodiester group through the 5' end of the antisense strand, where (a) the dsRNA was able to reduce gene expression, and (b) the dsRNA did not contain a free hydroxyl group on the 5' end of the antisense strand. It was not until this invention that the inventors recognized that a highly lipophilic group can be conjugated on the 5' end of the antisense strand in a manner that improved RNA interference activity and improved the biological activity of the dsRNA.

The examiner cites Fosnaugh as disclosing conjugation through the 5' end of the antisense strand. However, Appellants are not disputing that one skilled in art would not have contemplated the possibility of conjugating at the 5' end of the antisense strand. Fosnaugh was simply stating that the conjugates disclosed in this reference (not the conjugates recited in Appellants' claimed invention) can be attached at one of the ends of the strand.

Fosnaugh is also cited for the proposition that the conjugate can be attached through known biodegradable linkers, including phosphodiester linkages. But again, the focus of Fosnaugh lies in the conjugate, not any special function or relationship that the conjugate has to a particular point of attachment or a particular linkage.

Appellants respectfully submit that the examiner cannot simply combine the three other references with Fosnaugh for teaching (a) conjugation through the 5' end of the antisense strand and (b) the phosphodiester linkage, and arrive at Appellants' claimed invention. Fosnaugh provides boilerplate language listing a large number of alternatives with respect to the disclosed

conjugates. When taking into account the full disclosure of Fosnaugh, it becomes clear that there are numerous possibilities disclosed within the reference (or generally known by one of ordinary skill in the art) for choosing different linkages, different conjugates, and different ways to attach the conjugate to the dsRNA. However, there is no direction given why one skilled in the art would pursue the path identified by the examiner rather than the many other disclosed alternatives. Just because Fosnaugh states that the particular conjugates disclosed in that reference may be placed on any position in the dsRNA does not mean that the reference teaches that *any conjugate* can be placed in *any position* in the strand *with a reasonable expectation of success*. See MPEP § 2143.02 (a reasonable expectation of success is required; at least some degree of predictability is required to show obviousness).

Variation in even one of the variables when modifying the references can lead to any number of different compounds and not to those of the claimed invention. The examiner has not presented any rationale for showing how one of ordinary skill would navigate through each possibility disclosed by Fosnaugh, incorporating the alternatives that would work while disregarding those that would not work. Fosnaugh itself certainly provides no guidance on why certain positions of the dsRNA should be used for attachment or why certain linkages should be utilized.

The rejection set forth by the examiner follows a similar obviousness analysis that was rejected by the Federal Circuit in *Ortho-McNeil Pharm., Inc. v. Mylan Laboratories, Inc.*, 520 F.3d 1358 (Fed. Cir. 2008). In *Ortho-McNeil*, the Court found that Ortho-McNeil's compound topiramate was not obvious over known design choices for finding diabetes drugs. In reaching this conclusion, the Federal Circuit stated that Mylan's expert "simply retraced the path of the inventor with hindsight, discounted the number and complexity of the alternatives, and concluded that the invention of topiramate was obvious." *Id.* at 1364. Similarly, the examiner has not recognized the complexity and multitude of options available to Appellants at each or juncture in the process of preparing the dsRNA of the claimed invention.

In *Ortho-McNeil*, the Court stated that one of ordinary skill in the art would have to have some reason to select (among several unpredictable alternatives) the route that would ultimately lead to the claimed invention. 520 F.3d at 1364. The challenges of the inventive process would have prevented one of ordinary skill from traversing the multiple obstacles and arriving at the

claimed invention. *Id.* at 1365. In this case, like *Ortho-McNeil*, one of ordinary skill would have to have a rationale for selecting the particular route, amid unpredictable alternatives, that would have lead to the claimed invention. Appellants respectfully submit that such a rationale has not been shown by the examiner.

When confronted with the problem that Appellants faced before this invention, one skilled in the art would glean no useful information from Fosnaugh to assist in solving the recognized problem. Certainty, one skilled in the art would not interpret Fosnaugh as teaching that difficulties associated with conjugating through the 5' end of the antisense strand could be overcome by its broad, generic disclosures. See Appellants' Response filed February 12, 2010, describing how the prior art disfavors conjugation at the 5' end of the antisense strand, particularly in instances when a free hydroxyl group is not present at the 5' end.

Just because Fosnaugh discloses that conjugating may take place at both ends of both strands and just because that phosphodiester linkages are a type of linkage that can be used to conjugate does not mean that Fosnaugh provides the motivation for combining these two features to cure a problem in the art that Fosnaugh does not even mention, let alone recognize.

MPEP § 2143.01 states that the fact that references can be combined or modified does not render the resultant combination obvious unless the results would have been predictable to one of ordinary skill in the art. This is especially pertinent to the case at hand in view of the numerous reports disfavoring modifications at the 5' end of the antisense strand. The same section of the MPEP states that the mere statement that the claimed invention is within the capabilities of one of ordinary skill in the art is not sufficient by itself to establish *prima facie* obviousness.

Rejections of obviousness cannot be sustained by mere conclusory statements such as that offered by the examiner in the November 12, 2009 Office Action; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness. This is especially true in cases involving new chemical compounds, where it remains necessary to identify some reason that would have led a chemist to modify a known compound in a particular manner to establish *prima facie* obviousness. See *Takeda Chemical Indus., Ltd. v. Alphapharm Pty., Ltd.*, 492 F.3d 1350, 1357 (Fed. Cir. 2007).

In this case, Kay is the primary reference. Aside from being deficient in the two areas discussed above in the context of Fosnaugh (no disclosure relating to conjugation through the 5'

end of the antisense strand and no disclosure relating to a phosphodiester linkage), Kay additionally does not disclose a lipophilic conjugate. Nor does Fosnaugh for that matter. For the lipophilic group, the examiner relies on Frechet and Florence.

Frechet and Florence describe a lipophilic conjugate, in this case a dendrimer compound, that the examiner has substituted for the conjugate disclosed in Kay. According to the examiner, one skilled in the art would have wanted to incorporate a lipophilic group such as a dendrimer onto the dsRNA to mediate cellular uptake of the dsRNA more efficiently in methods of targeting HCV as taught by Kay. See Office Action mailed March 19, 2009, page 5.

The examiner then attempts to explain why these disclosures should be combined with Fosnaugh. To utilize the broad discloses of Fosnaugh, discussed above, the examiner states that "it would have been a design choice and a matter of routine experimentation." See Office Action mailed March 19, 2009, page 5. The motivation provided by the examiner ends there. The following discussion in the Office Action relates back to the motivation to utilize the dendrimers compounds and the benefits associated with lipophilic conjugates. See Office Action, pages 5-6.

Appellants respectfully submit that the examiner has not provided the detailed reasoning for why one skilled in the art would choose to conjugate this particular lipophilic compound (a) at the 5' end of the antisense strand, and (b) through a phosphodiester linkage. The problems associated with conjugating at the 5' end of the antisense strand--problems that have been well documented by Appellants--need to be keenly observed in making this analysis. One skilled in the art, knowing all the problems and difficulties in making this type of conjugation would be much less likely to try it. This is not the case where a reference recognizes the problem and teaches specific ways to overcome it. Fosnaugh is completely silent on this art-recognized problem; the 5' end of the antisense strand was simply provided as part of a comprehensive disclosure suggesting that Fosnaugh's particular conjugate could be attached through any means possible.

b. Rejection of claims 86, 94-98, and 110-119 under 35 U.S.C. § 103 as being unpatentable over the combination of Kay, Fosnaugh, Manoharan I, and Cook

The examiner has rejected claims 86, 94-98, and 110-119 as being unpatentable over Kay in view of Fosnaugh, Manoharan I, and Cook, as evidenced by Manoharan II.

As much of the basis for this rejection parallels the § 103 rejection discussed in section (a), *supra*, Appellants refer to those comments, particularly those comments directed towards Kay and Fosnaugh, in distinguishing the claimed invention over the cited prior art of this rejection.

There is an additional deficiency in this rejection, however, that merits attention. This deficiency was brought to the attention of the examiner by Applicants in the Response filed on February 12, 2010, but not addressed in the Advisory Action mailed March 11, 2010.

In the earlier § 103 rejection, the examiner states that neither Kay nor Fosnaugh taught a lipophilic group. See Office Action mailed March 19, 2009, page 4. The examiner relies on Frechet and Florence and their disclosure relating to the dendrimer compounds having a relatively high $\log K_{ow}$ value to teach this element of the claimed invention. See Office Action mailed March 19, 2009, pages 4-5.

In this rejection, the description of Kay and Fosnaugh is repeated, along with the acknowledgement that neither reference teach a lipophilic group. See Office Action mailed March 19, 2009, page 7. However, there are no secondary references that the examiner has cited teaching a lipophilic group having a $\log K_{ow}$ exceeding 1. This limitation is recited in claim 86, one of the rejected claims. Additionally, rejected claims 94-98, 110-111, and 114-119 are all dependent upon claim 86 and thus carry all of the claim limitations recited in claim 86.

While the examiner has cited Manoharan I, Cook, and Manoharan II to disclose lipophilic groups such as fatty acids, sterols, cholesterols, aromatic groups, and carbamate cholesterol groups (see Office Action mailed March 19, 2009, pages 7-9), there is no discussion of whether any of these lipophilic groups are also lipophilic groups having a $\log K_{ow}$ exceeding 1. As known in the art, certain sterols, steroids, and cholesterol derivatives are lipophilic to the degree where they have a $\log K_{ow}$ exceeding 1, while others are not. The examiner needs to show that the lipophilic compounds disclosed in Manoharan I, Cook, and Manoharan II meet the claim limitation relating to the $\log K_{ow}$ value exceeding 1, as recited in claim 86.

Without showing this, the rejection of claims 86, 94-98, 110-111, and 114-119 as being unpatentable over the combination of Kay, Fosnaugh, Manoharan I, and Cook and evidenced by Manoharan II does not teach or suggest every element of Appellants' claims.

c. Claims 100-102

Claims 100-102 are directed to dsRNAs where the lipophilic group has a $\log K_{ow}$ exceeding 1.5, exceeding 2, and exceeding 3, respectively. The examiner has rejected these claims based on the Frechet reference, showing lipophilic dendrimer groups, and Florence, allegedly showing that the dendrimer groups have a $\log K_{ow}$ of 17.5.

A closer analysis of Florence, however, reveals that the examiner cited an incorrect $\log K_{ow}$ value for this compound. On page 255 of Florence, the reference states "The partition coefficient of the dendrimer measured in an octanol/PBS (pH 7.4) was 17.5 and hence its log *P* value is 1.24." See top of p. 255 (carry-over paragraph from p. 254). The examiner misinterprets this passage as stating that the dendrimer has a $\log K_{ow}$ of 17.5, when it is actually stating that the log *P* value is 1.24. The 17.5 value is simply the partition coefficient without taking into account the "log" of this value. While Florence described the partition coefficient value as a log *P* and Appellants describe the value as $\log K_{ow}$, it is clear after reviewing both Appellants' specification and the complete Florence document that both terms are logs of the octanol-water partition coefficient. Compare Appellants' specification, p. 13, lines 3-21, describing the octanol-water partition coefficient with the abstract of Florence, describing the log *P* (octanol/water).

Because the dendrimer disclosed in Frechet and Florence has a octanol-water partition coefficient of less than 1.5 (1.24), these references do not suggest the additional lipophilic properties for compounds having $\log K_{ow}$ of greater than 1.5, as recited in claims 100-102.

In the Advisory Action mailed March 11, 2010, the examiner maintains the position that Florence teaches a $\log K_{ow}$ value of 17.5. According to the examiner, "the lipophilic group [of the claimed invention] is defined by the partition coefficient." However, the claims do not recite the partition coefficient, but the *log* of the partition coefficient, $\log K_{ow}$. In the same manner, Florence discloses that the partition coefficient of the dendrimer was 17.5, which equates to a log *P* value of 1.24. Whether the partition coefficient is described as *P* or K_{ow}, the logarithm of the ratio of the concentrations of the un-ionized solute in the solvents is the log *P* or the $\log K_{ow}$, the value recited in Applicants' claimed invention. While select portions of Applicants' specification may create some initial confusion regarding what the partition coefficient is, it is clear from a complete reading of the specification and myriad other publications defining the

partition coefficient that the partition coefficient is the K_{ow} value. The log of this partition coefficient, $\log K_{ow}$, is the widely used metric that Applicants have used in the claims.

Additionally, Appellants submit an article by Giri et al. entitled "Partitioning of Poly(amidoamine) Dentrimers between *n*-Octanol and Water" *Environ. Sci. Technol.* vol. 43, p. 5123-29 (2009) ("Giri")[1] showing that dendrimers are actually hydrophilic, not lipophilic. See Tables 1-3 on pages 5126-27, listing the $\log K_{ow}$ values at physiological pH 7.4 of the 18 PAMAM dendrimers evaluated. All tested dendimers produced negative $\log K_{ow}$ values, indicating to Giri that "these dendrimers are hydrophilic." See p. 5126. According to Giri, only a few measured values of $\log K_{ow}$ for dendrimers have been reported in the literature, including the Florence reference cited by the examiner. See p. 5125. At least one of these other references (Najlah), however, also reported dendrimers having negative $\log K_{ow}$ values. In view of this, Giri concluded, "These measurements corroborate our findings of negative $\log K_{ow}$ values for the 18 PAMAM dendrimers evaluated in this study." See p. 5126. Accordingly, despite the positive $\log K_{ow}$ value of the dendrimer reported in Florence, it seems that most of the literature has reported negative $\log K_{ow}$ values for dendrimers, indicating that dendrimers are <u>not</u> lipophilic.

In the Advisory Action, the examiner also states that "[t]here is nothing in either the specification or Florence that defines the log P and logKow as equivalent." However, if the terms are not equivalent, as the examiner suggests, it is still the examiner's burden to show that the claimed invention reads on the disclosed value in Florence. Florence discloses the log of the partition coefficient to be 1.24, whereas Applicants claim the log of the partition coefficient to be greater than 1.5 (in claims 100-102). Any which way this is analyzed, the claims do not read on the Florence value.

d. <u>Unexpected Results</u>

None of the references suggest the benefits Appellants have discovered associated with using lipophilic compounds having high $\log K_{ow}$ values. Using groups having increased lipophilic properties that are covalently linked to the dsRNA has enabled the dsRNA to exhibit

[1] In its discussion, Giri cites Florence, the reference applied by the examiner, making it pertinent to the rejection set forth by the examiner. While the Giri reference was not previously made of record, the clear teachings of this reference are in direct contrast to the conclusions reached by the examiner through Florence, Therefore, Giri should be made of record.

increased uptake by cells with or without a transfection aid. The derivatized dsRNA show surprisingly improved activity regardless of the mechanism of entry into the cell. Unlike similarly conjugated antisense RNA, the improved activity of the dsRNA of the claimed invention is independent of cellular association or receptor binding, and thus not a consequence of enhanced transport across cell membranes. See specification, page 17, lines 14-23.

Because of the highly lipophilic nature of these groups, Appellants have found that the dsRNA may be used "exclusively"; i.e. without auxiliary agents or encapsulating substances that might affect or mediate uptake of dsRNA in the cells that harbor the virus. Surprisingly, the inventors have discovered that compositions containing only naked dsRNA and physiologically acceptable solvent are taken up by cells, where the dsRNA effectively inhibits replication of the virus. The dsRNA of the claimed invention are thus particularly advantageous in that they do not require the use of an auxiliary agent to mediate uptake of the dsRNA into the cell, many of which agents are toxic or associated with deleterious side effects. See specification, page 22, line 18 to page 23, line 7.

In Fig. 3, Applicants provide various compounds conjugated with highly lipophilic groups. Four of these compounds, HCVC32-as, GalC32-as, HCVChol-as, and GalChol-as, illustrate examples of lipophilic groups covalently attached to a 5' end of the antisense strand in a dsRNA. See pages 32-33 of the specification. The lipophilic group cholesteryl (6-hyroxyhexyl) carbamate ("Chol") and 12-hydroxydodecanoic acid bisdecylamide ("C32") are specifically recited in claim 97. Fig. 3 demonstrates that dsRNA of the claimed invention, i.e. having one lipophilic group having a $\log K_{ow}$ exceeding one where the lipophilic group is covalently attached to the 5' end of the antisense strand and where the RNA strand contains a phosphodiester group, achieve an approximate 10-20% reduction in gene expression.

These advantages discovered by Appellants provide an example of the unexpected results exhibited by the claimed dsRNA covalently linked to highly lipophilic groups. Additionally, these advantages demonstrate the benefits associated with the lipophilic groups, in particular with regard to lipophilic groups having a $\log K_{ow}$ exceeding 1.0, and certainly for lipophilic groups having a $\log K_{ow}$ exceeding 1.5.

None of the references applied by the examiner disclose the benefits and surprising results discovered by Appellants associated with the lipophilic groups. The examiner must

consider these unexpected results in determining obviousness. See MPEP § 716.02(c) ("Evidence of unexpected results must be weighed against evidence supporting *prima facie* obviousness of the claimed invention.").

e. Conclusion

In view of the foregoing, it is clear that the rejections of the pending claims under 35 U.S.C. § 103(a) cannot be sustained. Accordingly, Appellants respectfully request that the Board reverse the rejection of claims 86, 94-98, 100-102, 110-111, and 114-119.

Respectfully submitted,

/Jeffrey N. Townes, Reg. No. 47,142/
Jeffrey N. Townes
Registration No. 47,142

Dated: May 21, 2010

Customer No. 84717
NIXON PEABODY LLP
Suite 900, 401 9th Street, N.W.
Washington, D.C. 20004-2128
202.585.8000

VIII. CLAIMS APPENDIX

86. A double-stranded ribonucleic acid (dsRNA) comprising a complementary RNA strand, a sense RNA strand and only one lipophilic group having a $\log K_{ow}$ exceeding 1, wherein the complementary RNA strand has a nucleotide sequence which is complementary to a target RNA, and wherein the target RNA is an mRNA transcript of a target gene or of a (+) strand RNA virus, wherein the lipophilic group is covalently attached to a 5'-end of the complementary RNA strand and a linkage between the lipophilic group and the 5'-end of the complementary RNA strand comprises a phosphodiester group.

94. The dsRNA of claim 86, wherein the lipophilic group is a steroid or a branched aliphatic hydrocarbon, or a combination thereof.

95. The dsRNA of claim 94, wherein the lipophilic group is a sterol.

96. The dsRNA of claim 95, wherein the sterol is cholesterol or a cholesterol derivative.

97. The dsRNA of claim 96, wherein the lipophilic group is cholesteryl (6-hydroxyhexyl) carbamate or 12-hydroxydodecanoic acid bisdecylamide.

98. The dsRNA of claim 86, wherein the lipophilic group is selected from the group consisting of an aromatic, aliphatic or alicyclic moiety, or a combination thereof.

100. The dsRNA of claim 86, wherein the lipophilic group has a $\log K_{ow}$ exceeding 1.5.

101. The dsRNA of claim 86, wherein the lipophilic group has a logKow exceeding 2.

102. The dsRNA of claim 86, wherein the lipophilic group has a logKow exceeding 3.

110. The dsRNA of claim 86, wherein the complementary RNA strand comprises a 3'-end and a 5'-end, and wherein the 3'-end has a nucleotide overhang of 1 to 4 nucleotides.

111. The dsRNA of claim 86, wherein the complementary RNA strand comprises a 3'-end and a 5'-end, and wherein the 3'-end has a nucleotide overhang of 1 or 2 nucleotides.

114. The dsRNA of claim 86, wherein the dsRNA is between 16 and 30 nucleotides in length.

115. The dsRNA of claim 86, wherein the dsRNA is between 16 and 25 nucleotides in length.

116. The dsRNA of claim 86, wherein the dsRNA is between 20 and 25 nucleotides in length.

117. The dsRNA of claim 86, wherein the target RNA is expressed in a cell selected from the group consisting of a hepatocyte, a pancreatic cell, a uterine cell, a cell of a cervix, and a cell of a urinary bladder.

118. The dsRNA of claim 86, wherein the (+) strand RNA virus is a Hepatitis C Virus (HCV).

119. The dsRNA of claim 86, wherein the target RNA is at least a portion of a 3'-untranslated region (3'-UTR) of a Hepatitis C Virus (HCV).

IX. EVIDENCE APPENDIX

There is no additional evidence being submitted in the Evidence Appendix.

X. RELATED PROCEEDINGS APPENDIX

There are no decisions rendered by a court or the Board on related appeals or interferences.

Environ. Sci. Technol. 2009, 43, 5123–5129

Partitioning of Poly(amidoamine) Dendrimers between *n*-Octanol and Water

JYOTSNENDU GIRI,[†] MAMADOU S. DIALLO,*[,†] WILLIAM A. GODDARD III,[†] NATHAN F. DALLESKA,[‡] XIANGDONG FANG,[§] AND YONGCHUN TANG[§]

Materials and Process Simulation Center, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA, Environmental Science and Engineering, Environmental Analysis Center, Department of California Institute of Technology, Pasadena, CA, and Power, Environmental, and Energy Research Center, Division of Chemistry and Chemical Engineering California Institute of Technology, Pasadena, CA

Received February 5, 2009. Revised manuscript received April 27, 2009. Accepted April 29, 2009.

Dendritic nanomaterials are emerging as key building blocks for a variety of nanoscale materials and technologies. Poly(amidoamine) (PAMAM) dendrimers were the first class of dendritic nanomaterials to be commercialized. Despite numerous investigations, the environmental fate, transport, and toxicity of PAMAM dendrimers is still not well understood. As a first step toward the characterization of the environmental behavior of dendrimers in aquatic systems, we measured the octanol–water partition coefficients ($\log K_{ow}$) of a homologous series of PAMAM dendrimers as a function of dendrimer generation (size), terminal group and core chemistry. We find that the $\log K_{ow}$ of PAMAM dendrimers depend primarily on their size and terminal group chemistry. For G1-G5 PAMAM dendrimers with terminal NH_2 groups, the negative values of their $\log K_{ow}$ indicate that they prefer to remain in the water phase. Conversely, the formation of stable emulsions at the octanol–water (O/W) interface in the presence of G6-NH_2 and G8-NH_2 PAMAM dendrimers suggest they prefer to partition at the O/W interface. In all cases, published studies of the cytotoxicity of Gx-NH_2 PAMAM dendrimers show they strongly interact with the lipid bilayers of cells. These results suggest that the $\log K_{ow}$ of a PAMAM dendrimer may not be a good predictor of its affinity with natural organic media such as the lipid bilayers of cell membranes.

Introduction

Nanotechnology has great potential to provide new and more effective functional materials to a broad range of industries including electronics, chemical, energy, water, food, and biomedical (*1*). Dendritic nanomaterials (NM), which include hyperbranched polymers, dendigrafts, dendronized polymers, dendrons and dendrimers, are emerging as key building blocks for a variety of nanoscale materials and technologies (*2*–*4*). Dendrimers are highly branched 3D globular nanostructures with controlled composition and architecture consisting of three components: a *core*, *interior branch cells*, and *terminal branch cells* (*3*, *4*). These are among the most versatile classes of NM available to date (*3*, *4*). These soft nanostructures, with sizes in the range of 2–20 nm, can be used as hosts for cations, anions and organic/inorganic solutes. Dendrimers can also be used as scaffolds and templates for the preparation of metal-encapsulated nanoparticles with tunable electronic, optical, and catalytic properties. They have also been successfully used as delivery vehicles or scaffolds for bioactive compounds. These unique properties of dendrimers are providing new opportunities for developing more effective functional materials for chemical separations and catalysis, medical imaging, drug delivery and water purification (*2*–*5*). As the United States Environmental Protection Agency (EPA) begins its assessment of the impact of nanotechnology on human health and the environment, there is a critical need for data and quantitative tools for assessing the environmental fate, transport and toxicity of NM such as dendrimers (*6*).

Poly(amidoamine) (PAMAM) dendrimers (Figure 1) were the first class of dendritic NM to be commercialized. The potential use of PAMAM dendrimers in biomedical applications such as gene therapy, drug delivery and magnetic resonance imaging has led many investigators to probe their biodistribution and toxicity. *In vitro* and *in vivo* (mice) toxicity studies show that dendrimer cytoxicity depends on concentration, generation and terminal group chemistry (*7*–*14*). Fuchs et al. (*9*) measured the *in vitro* toxicity of amine-based dendrimers (1.0–20.0 μM) using the human breast cancer MCF-7 cell line. The dendrimers evaluated consisted of G1 and G2 polyamidoamine dendrimers with various triamine cores and terminal groups including (1) quaternized amines, (2) benzyloxycarbonyl (boc) protecting groups, (4) boc protected and unprotected L-amino acids (phenylalanine, methionine, and aspartic acid), (5) diaminopropionic acid and (6) 5-dimethylamino-naphthlene-1-sulfonyl chloride (dansyl). They found that the dendrimers with neutral surface groups (i.e., dansyl groups) were nontoxic. Surprisingly, the dendrimers with positively charged diaminopropionic acid groups were also nontoxic in sharp contrast to a commonly accepted view that electrostatic interactions between protonated terminal groups of Gx-NH_2 PAMAM dendrimers and negatively charged head groups of lipid bilayers cause cell death by disrupting the integrity of cell membranes (*10*–*12*). More recently, Tisha et al. (*14*) have assessed the developmental toxicity of dendrimers using the zebrafish embryo. They found that a G4 PAMAM dendrimer with primary amine terminal groups attenuate the growth and development of zebrafish embryos at sublethal concentrations. In contrast, a G3.5 PAMAM dendrimer with carboxylic acid terminal groups, had no effect on the growth and development of the zebrafish embryos.

Despite these advances, the environmental fate and toxicity of dendritic NM such as PAMAM dendrimers is still not well understood. The partition coefficient ($\log K_{ow}$) of a compound between the isotropic solvent *n*-octanol and water is considered a measure of membrane affinity or lipophilicity. In environmental assessment, $\log K_{ow}$ is widely employed to predict the tendency of organic molecules to (i) sorb onto soil organic matter and (ii) bioaccumulate in the tissues of "living" media (e.g., zooplankton, benthic organisms, and fish) (*15*, *16*). Note that this classical approach may be not

* Corresponding author phone: 626 395 2730; fax: 626 585 0918.
[†] Materials and Process Simulation Center Division of Chemistry and Chemical Engineering.
[‡] Environmental Science and Engineering Division of Engineering and Applied Science.
[§] Power, Environmental, and Energy Research Center.

10.1021/es9003747 CCC: $40.75
Published on Web 05/19/2009

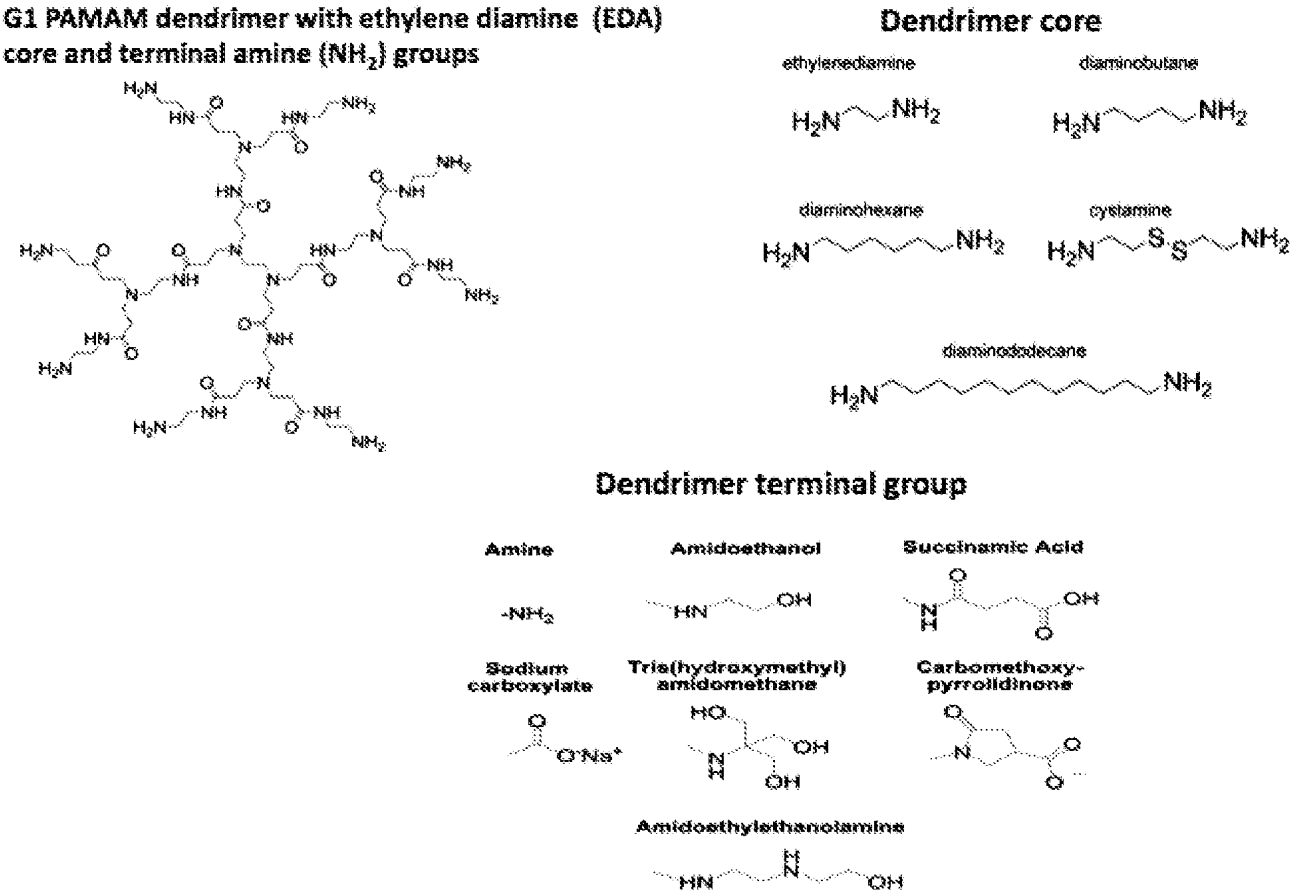

FIGURE 1. Core and terminal group chemistry of the PAMAM dendrimers evaluated in this study.

applicable to the assessment of the environmental fate and toxicity of NM such as carbon nanotubes, fullerenes, and metal oxide nanoparticles due to their tendency to aggregate in aqueous solutions; thus resulting in unpredictable bioavailability (*17*). Because dendrimer synthesis provides a variety of means for controlling molecular composition, size, shape, polydispersity, and solubility in aqueous solutions, dendrimers are ideal model systems for carrying out fundamental investigations of the environmental fate and toxicity of monodisperse and nonaggregated NM. As a first step toward the characterization of the environmental fate and behavior of dendrimers in aquatic systems, we initially focus on quantifying their partitioning between water and natural organic media using *n*-octanol as model system. We measured the $\log K_{ow}$ of a homologous series of 18 PAMAM dendrimers as a function of dendrimer generation (size), terminal group, and core chemistry. We find that the $\log K_{ow}$ of PAMAM dendrimers depend primarily on their size and terminal group chemistry. For G1-G5 PAMAM dendrimers with terminal NH_2 groups, the negative values of their $\log K_{ow}$ indicate that they prefer to remain in the water phase. Conversely, the formation of stable emulsions at the octanol–water (O/W) interface in the presence of G6-NH_2 and G8-NH_2 PAMAM dendrimers suggest they prefer to partition at the O/W interface. In all cases, published studies of the cytotoxicity of Gx-NH_2 PAMAM dendrimers show they strongly interact with the lipid bilayers of cells (*10*–*12*). These results suggest that the $\log K_{ow}$ of a PAMAM dendrimer may not be a good predictor of its affinity with natural organic media such as the lipid bilayers of cell membranes.

Materials and Methods

PAMAM dendrimers of different generations, core and terminal groups (Figure 1) were purchased (as methanol solutions or solids) from Dendritech, Sigma-Aldrich and Dendritic Nanotechnologies. Spectroscopic grade *n*-octanol and reagent grade trifluoroacetic acid were purchased from Sigma-Aldrich. OmniSolve acetonitrile was purchased from EMD.

Measurements of Octanol–Water Partition Coefficient ($\log K_{ow}$). The octanol–water coefficients ($\log K_{ow}$) of the PAMAM dendrimers were measured at room temperature using the standard "slow-stirring" method (*16*). Stock solutions of water-saturated octanol (WSO) and octanol-saturated water (OSW) were prepared by adding 450 mL of Milli-Q deionized water + 450 mL of octanol into a 1000 mL glass bottle with a tap at the bottom. The content of the bottle was mixed for 7 days by magnetic stirring using a Teflon-coated bar. The stirring speed was adjusted to create a vortex of ~2 cm at the octanol–water interface. Following equilibration and phase separation, the OSW was collected by decantation; whereas the WSO was collected through the bottle tap. The $\log K_{ow}$ measurements were carried out in 150 mL volumetric flasks. The concentration of each dendrimer was kept constant at 2.24 mM of equivalent terminal groups in all experiments unless otherwise specified. An aliquot of 25 mL of dendrimer + OSW was added to each flask. Following Diallo et al. (*18*), the pH of each dendrimer + OSW was adjusted to the desired value by addition of drops of concentrated HCl or NaOH. After pH adjustment, an aliquot of 90 mL of WSO was poured slowly against the upper wall of the flasks, which were then sealed with a threaded cap to minimize evaporation. The sample solutions and reference solutions (OSW + dendrimers) were placed and mixed at 160–180 rpm on a Corning magnetic stirrer. A picture of the experimental setup is shown in Supporting Information (SI) Figure S1.

Following equilibration, the concentrations of dendrimers in the OSW phase of each sample solution (C_d^w) and reference solution C_d^{total} were measured by high performance liquid chromatography (HPLC) (*19*). The HPLC system consisted of an Agilent 1100 vacuum degasser, a binary pump, an auto sampler and a column compartment with a UV diode array detector (G1315A). A Jupiter C5 silica-based reverse phase HPLC column (250 × 4.6 mm, 300 Å from Phenomenex, Torrance, CA) was used to separate the dendrimers. A Phenomenex Widepore C5 guard column (4 × 3 mm) was attached ahead of the analytical column. Milli-Q deonized water and acetonitrile (ACN) (50% v/v) were used as mobile phases. Trifluoracetic acid (0.14% v/v) was added to the mobile phases as ion pairing reagent and thus enabling dendrimer separation with a strictly reverse phase system. Typically, after a 5 min hold in 100% water, a linear gradient was employed from 0 to 50% ACN over 10 min followed by holding the system at 50% ACN water for 5 min. The injection volume was 25 μL with a solvent flow rate of 2 mL/minute. Dendrimer concentrations were determined by integration of the 210 nm chromatograms using Agilent's Chemstation software. The dendrimer concentrations in the WSO phase and reference solution were measured three times and averaged to determine C_d^o and C_d^w. The concentration of dendrimer in the octanol phase $C_d^o = (C_d^{total} - C_d^w)$ was determined by simple mass balance. logK_{ow} was expressed as follows:

$$\log K_{ow} = \frac{C_d^o}{C_d^W} \quad (1)$$

Results

To our knowledge, only a few measured values of logK_{ow} for dendrimers have been reported in the literature (*20, 21*). Thus, for the PAMAM dendrimers evaluated in this study, it was important to optimize the slow-stirring technique and HPLC assay to determine (1) the time required to reach equilibrium, (2) the optimum dendrimer concentration, and (3) the accuracy of the logK_{ow} measurements.

Measurements of Dendrimer Concentration by HPLC. In a previous article, Diallo et al. (*22*) showed that UV−vis spectroscopy (λ = 201 nm) can be used to measure the concentration of PAMAM dendrimers in aqueous solutions. Because *n*-octanol absorbs UV light at λ ~210 nm, our initial attempts to measure dendrimer concentration with UV in OSW solutions were unsuccessful. Thus, in this study, we used HPLC to measure dendrimer concentrations in the reference aqueous and OSW solutions. This technique exploits the ability of protonated PAMAM dendrimers to form ion pairs with trifluoroacetic acid (TFA) that can be sorbed onto and eluted from a C5 HPLC column. Note that the hydrophobicity of a PAMAM-TFA ion pair will depend on the size of the dendrimer and its number/type of terminal groups. This suggests that the elusion times of the sorbed PAMAM-TFA ion pairs will vary with generation and terminal group chemistry. SI Figures S2 and S3 show the effects of dendrimer generation and terminal group chemistry on elution time. For the Gx-NH_2 PAMAM dendrimers with ethylene diamine (EDA) core, we observed an increase in elution times with dendrimer generation (from 4.72 min for G1 to 7.3 min for G6). Similarly, the elution times of the nonamine terminated PAMAM dendrimers depend on terminal group chemistry: Tris- (t = 5.6 min), carboxylate (t = 6.3 min), amine (t = 6.3 min), succinamic acid (t = 6.9 min), amidoethanolamine (t = 6.9 min), pyrrolidinone (t = 8.7 min). Our results are consistent with those reported by Islam et al. (*19*). In all cases, the chromatograms are well resolved thereby suggesting HPLC is effective at separating PAMAM dendrimers. SI Figure S4 shows (as a typical example) that HPLC with UV detection (λ ~210 nm) can be used to quantify the concentration of PAMAM dendrimers in aqueous solutions. A series of standard solutions (10−60 μM of dendrimer) were used to generate the INSET calibration curve shown in SI Figure S4. Note that for all dendrimers evaluated in this study, we were able to detect and quantify very small differences (~1%) between the areas of their HPCL chromatograms in OSW solutions before and after equilibration with WSO solutions. SI Figure S5 shows a typical example of the integrated chromatographic areas of a G4 PAMAM dendrimer with succinamic acid groups in OSW before (area = 6051 ± 6 counts) and after equilibration [area = 5985 ± 2 counts]. In all cases, we measured the HPLC chromatogram areas three times to ensure that they were consistent and reproducible. We also found that the HPLC chromatograms become less resolved as dendrimer concentration decreases (SI Figure S4). Below a dendrimer concentration of 10 μM, the chromatograms of the PAMAM dendrimers were not well resolved. This suggests that the HPLC method is only effective at quantifying dendrimer concentrations greater than 10 μM using our experimental setup and measurement procedures.

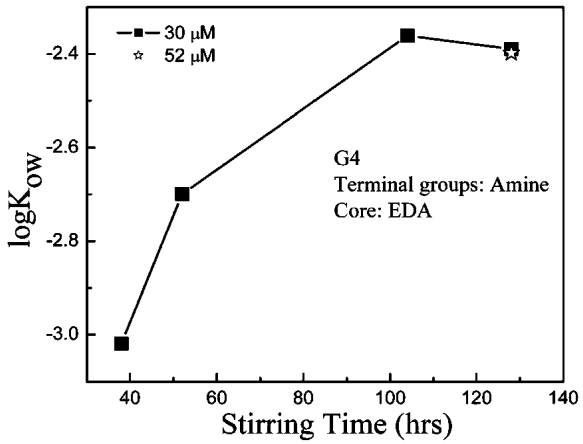

FIGURE 2. Effects of stirring time and dendrimer concentration on the logK_{ow} of G4-NH_2 PAMAM dendrimer at room temperature and pH 7.4.

Optimum Reaction Time, Dendrimer Concentration and Accuracy of Logk$_{ow}$ Measurements. Figure 2 illustrates the effect of reaction time and dendrimer concentration on the logK_{ow} of the G4-NH_2 PAMAM dendrimer. The logK_{ow} of this dendrimer reached constant value (−2.38) after 100 h of slow-stirring. De Bruijin et al. (*20*) have reported that the transfer of highly hydrophobic compounds (with logK_{ow}> 5) from *n*-octanol to water in slow-stirring experiments reached equilibrium in 4−5 days. Interestingly, we also found that the transfer of highly hydrophilic PAMAM dendrimers from water to *n*-octanol also reached equilibrium in 4−5 days. Thus, we have allowed 120 h slow-stirring in all experiments. Figure 2 also shows that the logK_{ow} of the G4-NH_2 PAMAM dendrimer remains constant as the concentration of dendrimer was increased from 30 to 52 μM. Thus, dendrimer concentration was kept constant at 2.24 mM of equivalent terminal groups in all subsequent logK_{ow} measurements. For example, this corresponds to a concentration of 34.0 μM for all G4 PAMAM dendrimers with 64 terminal groups. Because the logK_{ow} measurements required significant amounts of dendrimers, we only carried out three replicate measurements in selected cases to estimate an upper limit for the accuracy of the logK_{ow} measurements. However, we used at least two replicate in all cases. Note that the logK_{ow} measurements were reproducible in all cases (Figures 3−6). We subsequently selected three generation (G4) with different terminal groups (amine, succinamic acid and pyrrolidinone) and carried out three replicate logK_{ow} measurements for these dendrimers. These replicate measurements and average

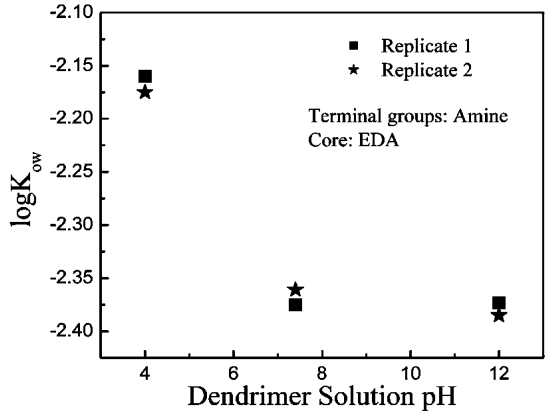

FIGURE 3. Effects of solution pH on the logK_{ow} of G4-NH_2 PAMAM dendrimer at room temperature and pH 7.4.

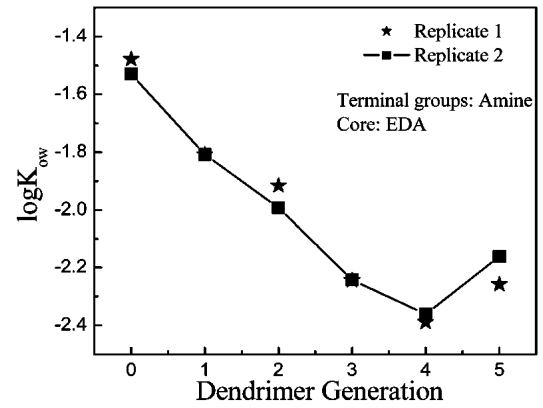

FIGURE 4. Effects of generation on the logK_{ow} of Gx-NH_2 PAMAM dendrimers at room temperature and pH 7.4.

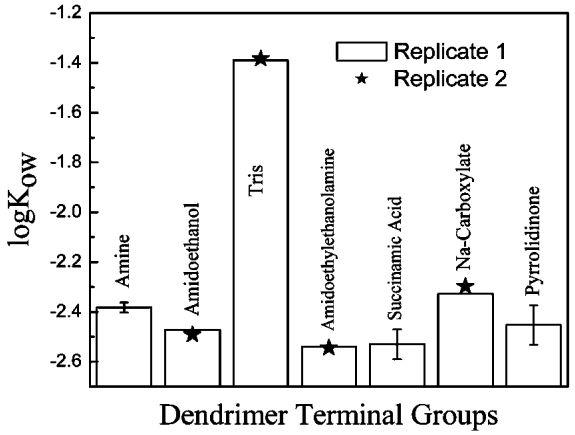

FIGURE 5. Effects of dendrimer terminal group chemistry on the logK_{ow} of G4 PAMAM dendrimers (G3.5 for Na-Carboxylate) at room temperature and pH 7.4. All the dendrimers have an EDA core except the G4 dendrimer with amidoehylethanolamine terminal groups which has a DAB core.

logK_{ow} are given in Table 2. The corresponding standard deviations are, respectively, equal to 0.02, 0.06, and 0.08 for the G4 PAMAM dendrimers with amine, succinamic acid, and pyrrolidinone terminal groups. Based on the results of these replicate experiments, we estimate the accuracy of the logK_{ow} of the PAMAM dendrimers evaluated in this study to be $\sim \pm 0.10$.

Effects of pH on logK_{ow}. We assessed the effect of solution pH on the logK_{ow} of PAMAM dendrimers using the G4-NH_2 dendrimer as model system. Figure 3 shows the logK_{ow} values of the G4-NH_2 dendrimer in acidic (pH 4.0), neutral (pH 7.4) and basic (ph 12.0) OSW solutions. Note that this dendrimer has higher (less negative) logK_{ow} (−2.15) at acidic pH (4) than at neutral (7.4) and basic pH (12). There is no significant change in the dendrimer logK_{ow} (−2.38) when pH changes from neutral to basic.

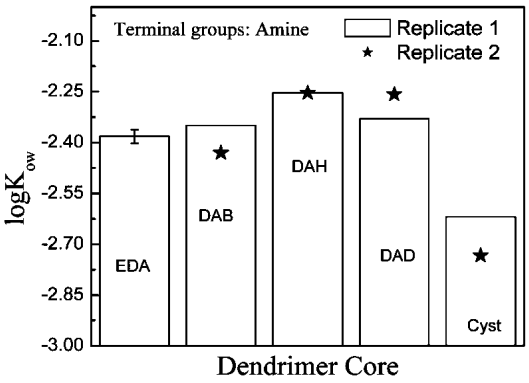

FIGURE 6. Effects of dendrimer core chemistry on the logK_{ow} of G4 PAMAM dendrimer at room temperature and pH 7.4.

TABLE 1. Octanol−Water Partition Coefficients (logK_{ow}) of PAMAM Dendrimers with Ethylene Diamine (EDA) Core and Terminal NH_2 Groups at Room Temperature and pH 7.4

generation	logK_{ow}1	logK_{ow}2	logK_{ow}3	logK_{ow} (reported)
G0	−1.48	−1.53		−1.53
G1	−1.81	−1.81		−1.81
G2	−1.91	−1.99		−1.99
G3	−2.24	−2.24		−2.24
G4	−2.39	−2.36	−2.39	−2.38 ± 0.02
G5	−2.26	−2.16		−2.16
G6				[a]ND
G8				[a]ND

[a] ND: not determined. This dendrimer caused the formation of an emulsion at octanol−water interface (see SI Figure S6).

Effects of Dendrimer Generation, Terminal Group and Core Chemistry on logK_{ow} at pH 7.4. Tables 1, 2, and 3 list the logK_{ow} values at physiological pH 7.4 of the 18 PAMAM dendrimers (Figure 1) evaluated in this study. Note that the logK_{ow} of the dendrimers are all negative thereby suggesting these dendrimers are hydrophilic. Figure 4 shows that the logK_{ow} of Gx-NH_2 PAMAM dendrimers gradually decrease with generation/size (i.e., become more negative) from generation 1 (G1) to generation 4 (G4) followed by a slight increase at generation 5 (G5). To our knowledge, only a limited number of measured logK_{ow} values for PAMAM dendrimers have published. Najlah et al. (*20*) reported that G1-NH_2 PAMAM dendrimers functionalized with two different terminal hydrophobic groups (terfenadine-succinyl and terfenadine-succinyl-diethylene glycol) have negative logK_{ow} values (−0.16). These measurements corroborate our finding of negative logK_{ow} values for the 18 PAMAM dendrimers evaluated in this study. Figure 5 and Table 2 illustrate the effect of dendrimer terminal chemistry on the logK_{ow} of PAMAM dendrimers. We tested seven different dendrimer terminal groups: cationic (amine, amidoethylethanolamine), anionic (carboxylate, succinamic acid, and pyrrolidinone), and neutral (amidoethanol and tris(hydroxymethyl)amidomethane (Tris)). Note that the G4 PAMAM dendrimer with Tris terminal groups has the highest (less negative) logK_{ow} (−1.39). Figure 6 and Table 3 show the variation of logK_{ow} values of G4-NH_2 dendrimer with different core. There is a slight increase of the logK_{ow} values of the G4-NH_2 PAMAM dendrimers as the number of their core carbon atoms increase from 2 (EDA) to 6 (DAH).

TABLE 2. Octanol–Water Partition Coefficients ($\log K_{ow}$) of PAMAM Dendrimers with Different Terminal Groups at Room Temperature and pH 7.4

generation	terminal group	$\log K_{ow}1$	$\log K_{ow}2$	$\log K_{ow}3$	$\log K_{ow}$ (reported)
4	amine	−2.39	−2.36	−2.39	−2.38 ± 0.02
4	amidoethanol	−2.47	−2.49		−2.47
4	succinamic acid	−2.53	−2.59	−2.48	−2.53 ± 0.06
3.5	sodium carboxylate	−2.30	−2.33		−2.33
4	tris(hydroxymethyl) aminomethane	−1.39	−1.39		−1.39
4	pyrrolidinone	−2.55	−2.42	−2.39	−2.45 ± 0.08
4	amidoethylethanolamine	−2.54	−2.54		−2.54

TABLE 3. Octanol–Water Partition Coefficients ($\log K_{ow}$) of G4 PAMAM Dendrimers with Different Cores at Room Temperature and pH 7.4

core[a]	terminal group	$\log K_{ow}1$	$\log K_{ow}2$	$\log K_{ow}3$	$\log K_{ow}$ (reported)
EDA	amine	−2.39	−2.36	−2.39	−2.38 ± 0.02
DAB	amine	−2.43	−2.35		−2.35
DAH	amine	−2.25	−2.25		−2.25
DAD	amine	−2.26	−2.33		−2.33
Cyst	amine	−2.62	−2.73		−2.62

[a] EDA (ethylene diamine); DAB (diaminobutane); DAH (diaminohexane); DAD (diaminododecane), and Cyst (cystamine).

Discussion

Due to the wide utilization of $\log K_{ow}$ in drug design, medicinal chemistry, and environmental assessment, a database of over 60 000 measured values of $\log K_{ow}$ for organic molecules has been developed by the Biobyte Corporation (*24*). Several methods of estimations of $\log K_{ow}$ from "structure" have also been developed during the last 20 years. Quantitative-structure activity relationships (QSAR) of estimation of $\log K_{ow}$ include the fragment-based methods (CLOGP) of Hansch and Leo (*25*), the extended group contribution method (KLOGP) of Klopman et al. (*26*), and the atomic constant approach (ALOGP) of Goshe et al. (*27*). Although these QSAR have been shown to reproduce very well the $\log K_{ow}$ values of their training data sets, there are several classes of solutes such as peptides and nucleosides which often cause them "difficulties". To quote Leo "when CLOGP fails badly, there are strong indications that it is conformational information that is lacking" (*25*).

The G4-NH_2 PAMAM dendrimer provides a good model system for explaining the effect of solution pH and macromolecular conformation on the octanol–water partitioning behavior of PAMAM dendrimers. First, a G4-NH_2 PAMAM dendrimer exhibits three distinct charged states in water as a function of solution pH (*18*). At acidic pH (<5), all the primary and tertiary amine groups of a G4-NH_2 PAMAM dendrimer are protonated. At neutral pH (~7.0), only the primary amine groups of this dendrimer are protonated. Conversely, all the amine groups of the G4-NH_2 PAMAM become unprotonated and neutral at basic pH (>10.0). Figure 7 shows the 3D structures of a G4-NH_2 PAMAM in water with Cl^- counterions at these three distinct protonation levels as determined from atomistic molecular dynamics (MD) simulations by Yi et al. (*28*). By using a modified Dreiding force field with interaction potentials that were fitted to high level density functional theory (DFT) calculations, Yi et al. (*28*) obtained calculated radii of gyration (R_g) (~2.11–2.20 nm) that were in excellent agreement with estimated R_g from small angle neutron scattering (SANS) experiments (*29*). Note that the G4 PAMAM dendrimer undergoes drastic conformational changes as solution pH decreases (Figure 7). Although the R_g of the G4-NH_2 PAMAM dendrimer does not vary with pH, Yi et al. (*28*) found that a decrease in solution pH causes a redistribution of mass within the G4 PAMAM dendrimer as its conformation changes from a "dense core" at high pH to a "dense shell" at low pH. They also found that the backfolding of the terminal NH_2 groups of the G4 PAMAM is mainly localized at the dendrimer periphery. Thus, the conformational flexibility of the G4 PAMAM dendrimer in aqueous solutions of different pH strongly suggest that QSAR based on group contributions (e.g., CLOGP, KLOGP, and ALOGP) cannot provide reliable estimates of $\log K_{ow}$ for NM such as dendrimers.

At the present time, we do not have a quantitative atomistic model for explaining the effect of solution pH on the $\log K_{ow}$ of the G4-NH_2 PAMAM dendrimer. However, this effect could be attributed to a competition between the electrostatic and cavity contributions to the free energy of transfer of a dendrimer from water to *n*-octanol. The $\log K_{ow}$ (i) of a nonassociating solute "*i*" can be expressed as follows (*30*):

$$\log K_{ow} = \frac{-\Delta G_W^o(i)}{2.30RT} \tag{2}$$

$$\Delta G_W^o(i) = \Delta G_o(i) - \Delta G_w(i) \tag{3}$$

Where $\Delta G_W^o(i)$ is the free energy of transfer of solute "*i*" from water to *n*-octanol; $\Delta G_o(i)$ and $\Delta G_w(i)$ are, respectively, the solvation free energies of "*i*" in *n*-octanol and water; T is the temperature and R is the ideal gas constant. Macroscopic solvent models can provide simple, fast, and reliable means of calculating the solvation energies of biological macromolecules such as proteins (*31*). These models treat the solvent as a dielectric continuum and the solute as "low dielectric" molecular cavity with point charges placed on the atomic nuclei of its van der Waals surface. The basic premise of a macroscopic solvent model is that the electrostatic and nonpolar contributions to the solvation free energy of solute can be evaluated separately. Following Honig et al. (*30*), the solvation free energy of solute "*i*" in solvent "*s*" [$\Delta G_o(i)$] can be expressed as follows:

$$\Delta G_s(i) = \Delta G_s^{el}(i) + \Delta G_s^c(i) \tag{4}$$

$$\Delta G_s^c(i) = \gamma_s A_s(i) \tag{5}$$

where $\Delta G_s^{el}(i)$ and $\Delta G_s^c(i)$ are, respectively, the electrostatic and cavity (i.e., nonpolar) contributions to the free energy of solvation of solute in solvent s; γ_s and A_s are, respectively, the microscopic surface tension and solvent-accessible surface area of solute "*i*" in solvent "*s*". Substituting eqs 3, 4, and 5 into eq 2 yields

$$\log K_{ow} = \frac{[\Delta G_W^{el}(i) + \gamma_w A_w(i)] - [\Delta G_o^{el}(i) + \gamma_o A_o(i)]}{2.30RT} \tag{6}$$

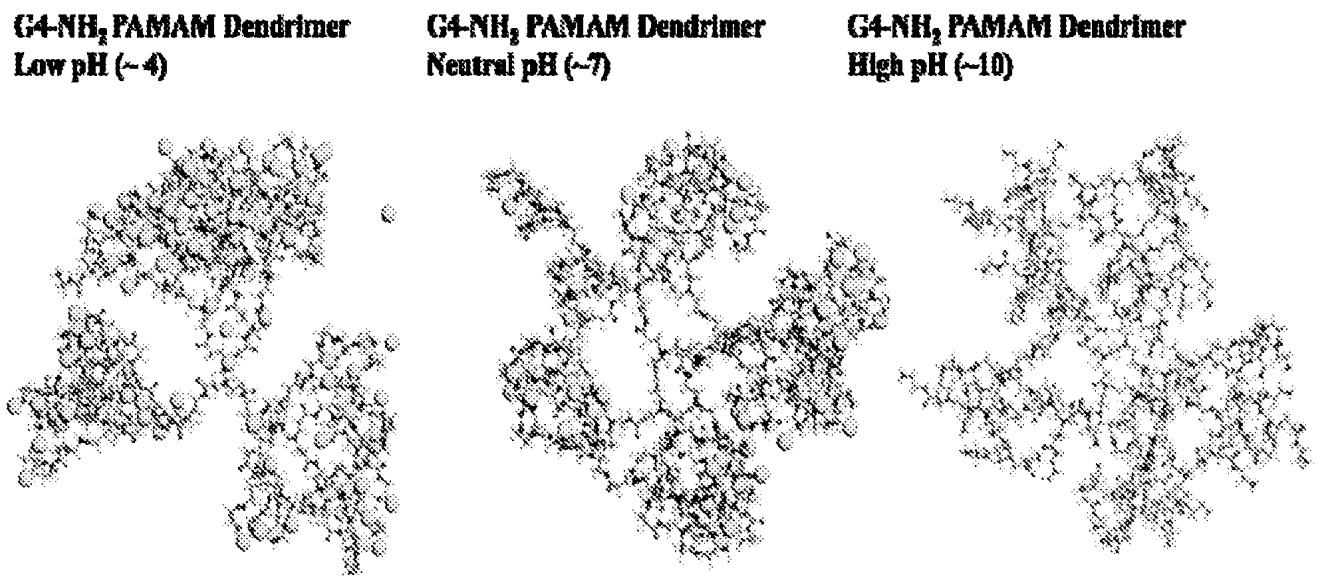

FIGURE 7. Three-dimensional structures of a G4-NH_2 PAMAM dendrimer in water (at low, neutral, and high pH) from atomistic molecular dynamics (MD) simulations (see ref *28*). The green atoms are bound Cl^- counterions that neutralize the protonated primary and tertiary amine groups of the dendrimer.

Equation 6 provides a thermodynamic framework for explaining the effect of solution pH on $\log K_{ow}$ of the G4-NH_2 PAMAM dendrimer. First, the negative values of the $\log K_{ow}$ of PAMAM dendrimers (Tables 1−3) indicate that dendrimer hydration free energy $[\Delta G_w^{el}(i) + \gamma_w A_w(i)]$ is very favorable (i.e., smaller than dendrimer solvation free energy in the octanol phase). Recall that at pH 4.0, all the primary and tertiary amine groups of a G4-NH_2 PAMAM dendrimer are protonated (*18*). However, SANS experiments by Chen et al. (*29*) and atomistic MD simulations by Liu et al. (*28*) (Figure 7) have established that a significant fraction (>66%) of the protonated amine groups of a G4 PAMAM dendrimer become neutralized by bound Cl^- counterions at acid pH (<5). Moreover Liu et al. (*28*) have found that the solvent (water) accessible of the G4 PAMAM dendrimer is lower in magnitude (by ~15%) than at neutral pH. Thus, we hypothesize that the solvation free energy of the G4 PAMAM dendrimer in water $[\Delta G_w(i)]$ to become less favorable as its cavity term $\Delta G_s^c(i)$ decrease in magnitude and its electrostatic component $\Delta G_w^{el}(i)$ becomes less negative due the neutralization of its protonated amine groups by bound Cl^- anions at pH 4.0.

Figure 3 and Table 1 show a slight increase of $\log K_{ow}$ for the G5 PAMAM dendrimer (−2.16) compared to that of the G4 PAMAM dendrimer (−2.38). SI Figure S6 shows that the G6-NH_2 PAMAM dendrimer causes the formation of emulsions as illustrated by the cloudiness of the octanol−water (O/W) interface. We hypothesized this increased affinity for the O/W interface of the G5-NH_2, G6-NH_2, and G8-NH_2 PAMAM dendrimers may be the result of changes in their hydrophilic−lipophilic balance due to global and local conformational events within the macromolecules. First, Gx-NH_2 PAMAM dendrimers undergo a gradual transition in overall shape (from a more extended conformation for "earlier" generation dendrimers (G ≤ 3.0) to a more compact/globular shape for "later" generation dendrimers (G ≥ 5.0)]) (*31*). Second, atomistic MD simulations with explicit water molecules by Yi et al. (*28*) (Figure 7) have shown that the backfolding of the terminal NH_2 groups of PAMAM dendrimers at neutral pH is mainly localized at the dendrimer periphery. Thus, it is reasonable to postulate that global and local conformational changes within the more compact and sterically crowded G6-NH_2 and G8-NH_2 PAMAM dendrimers could cause these macromolecules to act as amphiphiles at the O/W interface. This hypothesis is consistent with recent experiments showing that homologues of *p*-methyl benzyl alkylamines also undergo conformational changes and accumulate at the octanol−water interface (*32*). However, independent experimental measurements and atomistic MD simulations will be needed to corroborate this hypothesis.

Except for the PAMAM dendrimer with sodium carboxylate terminal group (G3.5), all dendrimers were fourth generation (G4) and thus have similar size and same number of tertiary amine and amide groups. Note that the highest (i.e., less negative) $\log K_{ow}$ value (−1.39) for the G4 PAMAM dendrimer with Tris terminal groups may be attributed due to its large number of terminal OH groups (64 × 3 = 192). Thus, its increased affinity for the octanol phase could be attributed to increased H-bonding between the G4-Tris dendrimer macromolecules and *n*-octanol molecules. The $\log K_{ow}$ values of all the other G4 PAMAM dendrimers are comparable in magnitude ranging from −2.54 to −2.33. Not surprisingly, the $\log K_{ow}$ of the G4-NH_2 PAMAM with EDA, DAB, DAH, and DAD cores are also comparable ranging from −2.38 to −2.25. However, the G4-NH_2 PAMAM with cystamine core has the lowest $\log K_{ow}$ value (−2.62). At the present time, we do not have a quantitative explanation for this observation.

Environmental Implications

As previously stated, the logarithm of the partition coefficient of an organic compound between *n*-octanol and water ($\log K_{ow}$) is one of the most widely used measures of its tendency to accumulate in natural biomedia such as the lipid membranes of animals, plants, and bacteria (*15*). Several investigators have shown that the lipid−water coefficients ($\log K_{lipw}$) of many polar and nonpolar organic pollutants are positively and often linearly correlated with their $\log K_{ow}$ (*15*). Because of these correlations, $\log K_{ow}$ is often employed as descriptor in QSAR used to evaluate the baseline toxicity of xenobiotic organic compounds as they accumulate in natural biomedia such as lipid bilayers (*15*). However, to our knowledge, correlations between the $\log K_{ow}$ of organic nanomaterials such as dendrimers and their accumulation in natural biomedia have not been established. Note that the G1-G5 PAMAM dendrimers with terminal NH_2 groups evaluated in this study (Figure 1) have negative $\log K_{ow}$ values thereby suggesting they prefer to remain in water (Table 1). Conversely, the formation of stable emulsions at the octanol−water (O/W) interface in the presence of G6-NH_2 and G8-NH_2 PAMAM dendrimers suggest they prefer to

partition at the O/W interface (SI Figure S6). However, published atomic force microscopy (AFM) imaging and cytotoxicity studies show that Gx-NH_2 PAMAM dendrimers strongly interact with the lipid bilayers of cells (*10*–*12*). This suggests that logK_{ow} of a PAMAM dendrimer may not be a good predictor of its affinity with natural organic media such as the lipid bilayers of cell membranes.

Acknowledgments

This work was carried out in the Materials Process Simulation Center and Power, Environmental and Energy Research Center of the Division of Chemistry and Chemical Engineering at the California Institute of Technology. Funding was provided by the Environmental Protection Agency (EPA STAR Grant RD832525) and the National Science Foundation (NIRT CBET Award No. 050695).

Supporting Information Available

Further details are shown in three tables and six figures. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) U.S. NNI. http://www.nano.gov/html/facts/nanoapplicationsandproducts.html.
(2) *Dendrimers and Other Dendritic Polymers*; Fréchet, J. M. J., Tomalia, D. A., Eds.; Wiley and Sons: New York, 2001.
(3) Helms, B.; Meijer, E. W. Dendrimers at work. *Science.* **2006**, *313*, 929–930.
(4) Tomalia, D. A., Henderson, S. A.; Diallo, M. S. Dendrimers—An enabling synthetic science to controlled organic nanostructures. In *Handbook of Nanoscience, Engineering and Technology*, Chapter 24, 2nd ed.; Goddard, W. A. III.; Brenner, D. W.; Lyshevski, S. E. and Iafrate, G. J.; Eds.; CRC Press: Boca Raton, FL, 2007.
(5) Diallo M. S. Water treatment by dendrimer-enhanced filtration: Principles and applications. In *Nanotechnology Applications for Clean Water*; Savage, N., Diallo, M. S, Duncan, J., Street, S. R., Eds.; William Andrew Applied Science Publishers: Norwich, 2009.
(6) Tinkle, S. S. Nanotechnology: collaborative opportunities for ecotoxicology and environmental health. *Environ. Toxicol. Chem.* **2008**, *27*, 1823–1824.
(7) Roberts, J. C.; Bhalgat, M. K.; Zera, R. T. Preliminary biological evaluation of polyamidoamine (PAMAM) Starburst® dendrimers. *J. Biomed. Mater. Res.* **1996**, *30*, 53–65.
(8) Malik, N.; Wiwattanapatapee, R.; Klopsch, R.; Lorenz, K.; Frey, H.; Weener, J. W.; Meijer, E. W.; Paulus, W.; Duncan, R. Dendrimers: Relationship between structure and biocompatibility in vitro, and preliminary studies in the biodistribution of 125I-labelled polyamidoamine dendrimers in vivo. *J. Controlled Release* **2000**, *65*, 133–148.
(9) Fuchs, S.; Kapp, T.; Otto, H.; Schoneberg, T.; Franke, P.; Gust, R.; Schluter, A. D. A Surface-modified dendrimer set for potential application as drug delivery vehicles: synthesis, in vitro toxicity and intracellular localization. *Chem.—Eur. J.* **2004**, *10*, 1167–1192.
(10) Hong, S. P.; Bielinska, A. U.; Mecke, A.; Keszler, B.; Beals, J. L.; Shi, X. Y.; Balogh, L.; Orr, B. G.; Baker, J. R.; Holl, M. M. B. Interaction of poly(amidoamine) dendrimers with supported lipid bilayers and cells: hole formation and the relation to transport. *Bioconjugate Chem.* **2004**, *15*, 774–782.
(11) Leroueil, P. R.; Hong, S. Y.; Mecke, A.; Baker, J. R.; Orr, B. G.; Holl, M. M. B. Nanoparticle interaction with biological membranes: Does nanotechnology present a janus face? *Acc. Chem. Res.* **2007**, *40*, 335–342.
(12) Leroueil, P. R.; Berry, S. A.; Duthie, K.; Han, G.; Rotello, V. M.; McNerny, D. Q.; Baker, J. R.; Orr, B. G.; Holl, M. M. B. Wide varieties of cationic nanoparticles induce defects in supported lipid bilayers. *Nano Lett.* **2008**, *8*, 420–424.
(13) Metullio, L.; Ferrone, M.; Coslanich, A.; Fuchs, S.; Fermeglia, M.; Paneni, M. S.; Pricl, S. Polyamidoamine (Yet not PAMAM) dendrimers as bioinspired materials for drug delivery: sStructure-activity relationships by molecular simulations. *Biomacromolecules.* **2004**, *5*, 1371–1378.
(14) Heiden, T. C. K.; Dengler, E.; Kao, W. J.; Heideman, W.; Peterson, R. E. Developmental toxicity of low generation PAMAM dendrimers in zebrafish. *Toxicol. Appl. Pharmacol.* **2007**, *225*, 70–79.
(15) Schwarzenbach, R. P.; Gschwend, P.; Imboden, D. M. *Environmental Organic Chemistry*, 2nd.; John Wiley & Sons, Inc: New York, 2003.
(16) Sangster, J. *Octanol-Water Partition Coefficients: Fundamentals and Physical Chemistry*, Wiley Series in Solution Chemistry; John Wiley & Sons: New York, 1997; Vol. 2.
(17) Klaine, S. J.; Alvarez, P. J. J.; Batley, G. E.; Fernandes, T. F.; Handy, R. D.; Lyon, D. Y.; Mahendra, S.; Mclaughlin, M. J.; Lead, J. R. Nanomaterials in the environment: Behavior, fate, bioavailability and effects. *Environ. Toxicol. Chem.* **2008**, *27*, 1825–1851.
(18) Diallo, M. S.; Christie, S.; Swaminathan, P.; Balogh, L.; Shi, X.; Um, W.; Papelis, L.; Goddard, W. A. III.; Johnson, J. H. Jr. Dendritic chelating agents 1. Cu(II) binding to ethylene diamine core poly(amidoamine) dendrimers in aqueous solutions. *Langmuir.* **2004**, *20*, 2640–2651.
(19) Islam, M. T.; Shi, X. Y.; Balogh, L.; Baker, J. R. HPLC separation of different generations of poly(amidoamine) dendrimers modified with various terminal groups. *Anal. Chem.* **2005**, *77*, 2063–2070.
(20) Najlah, M.; Freeman, S.; Attwood, D.; D'Emanuele, A. Synthesis and assessment of first-generation polyamidoamine dendrimerprodrugs to enhance the cellular permeability of P-gp substrates. *Bioconjugate Chem.* **2007**, *18*, 937–946.
(21) Florence, A. T.; Sakthivel, T.; Toth, I. Oral uptake and translocation of a polylysine dendrimer with a lipid surface. *J. Controlled Release* **2000**, *65*, 253–259.
(22) Diallo, M. S.; Christie, S.; Swaminathan, P.; Johnson, J. H., Jr; Goddard, W. A. III. Dendrimer enhanced ultrafiltration. 1. Recovery of Cu(II) from aqueous solutions using Gx-NH_2 PAMAM dendrimers with ethylene diamine core. *Environ. Sci. Technol.* **2005**, *39*, 1366–1377.
(23) De Bruijn, J.; Busser, F.; Seinen, W.; Hermens, J. Determination of octanol/water partition coefficients for hydrophobic organic chemicals with the 'slow-stirring' method. *Environ. Sci. Technol.* **1989**, *8*, 499–512.
(24) Bio-Loom. Available at http://www.biobyte.com/bb/prod/bioloom.html
(25) Leo, A. J. Calculating log P_{oct} from structures. *Chem. Rev.* **1993**, *83*, 1281–1306.
(26) Sedykh, A. Y.; Klopman, G. Structural analogue approach to the prediction of the octanol-water partition coefficient. *J. Chem. Inf. Model.* **2006**, *46*, 1598–1603.
(27) Goshe, A. K.; Wishwanadhan, V. N.; Wendoloski, J. J. Prediction of hydrophobic (Lipophilic) properties of small organic molecules using fragment methods: an analysis of ALOGP and CLOGP methods. *J. Phys. Chem. A.* **1998**, *102*, 3762–3772.
(28) Liu, Yi.; Bryantsev, V. S.; Diallo, M. S.; Goddard, W. A. III. PAMAM dendrimers undergo pH responsive conformational changes without swelling. *J. Am. Chem. Soc.* **2009**, *131* (8), 2798–2799.
(29) Chen, W. R.; Porcar, L.; Liu, Y.; Butler, P. D.; Magid, L. J. Small angle neutron scattering studies of the counterion effects on the molecular conformation and structure of charged G4 PAMAM dendrimers in aqueous solutions. *Macromolecules.* **2007**, *40*, 5887–5898.
(30) Honig, B.; Sharp, K.; Yang, A. S. Macroscopic models of aqueous-solutions - biological and chemical applications. *J. Phys. Chem.* **1993**, *97*, 1101–1109.
(31) Bosman, A. W.; Janssen, H. M.; Meijer, E. W. About Dendrimers: Structure, physical properties and applications. *Chem. Rev.* **1999**, *99*, 1665–1668.
(32) Fruttero, R.; Caron, G.; Fornatto, E.; Boschi, D.; Ermondi, G.; Gasco, A.; Carrupt, P.; Testa, B. Mechanisms of liposomes/water partitioning of (p-methylbenzyl) alkylamine. *Pharm. Res.* **1998**, *15*, 1407–1413.

ES9003747